JOURNAL OF BACTERIOLOGY, Aug. 1997, p. 4821–4830
0021-9193/97/$04.00+0
Copyright © 1997, American Society for Microbiology
Vol. 179, No. 15

Cloning and Analysis of the Poly(3-Hydroxybutyrate-*co*-3-Hydroxyhexanoate) Biosynthesis Genes of *Aeromonas caviae*

TOSHIAKI FUKUI AND YOSHIHARU DOI*

Polymer Chemistry Laboratory, Institute of Physical and Chemical Research (RIKEN), Hirosawa, Wako-shi, Saitama 351-01, Japan

Received 13 December 1996/Accepted 14 April 1997

A 5.0-kbp *Eco*RV-*Eco*RI restriction fragment was cloned and analyzed from genomic DNA of *Aeromonas caviae*, a bacterium producing a copolyester of (*R*)-3-hydroxybutyrate (3HB) and (*R*)-3-hydroxyhexanoate (3HHx) [P(3HB-*co*-3HHx)] from alkanoic acids or oils. The nucleotide sequence of this region showed a 1,782-bp poly (3-hydroxyalkanoate) (PHA) synthase gene ($phaC_{Ac}$ [i.e., the *phaC* gene from *A. caviae*]) together with four open reading frames (ORF1, -3, -4, and -5) and one putative promoter region. The cloned fragments could not only complement PHA-negative mutants of *Alcaligenes eutrophus* and *Pseudomonas putida*, but also confer the ability to synthesize P(3HB-*co*-3HHx) from octanoate or hexanoate on the mutants' hosts. Furthermore, coexpression of ORF1 and ORF3 genes with $phaC_{Ac}$ in the *A. eutrophus* mutant resulted in a decrease in the polyester content of the cells. *Escherichia coli* expressing ORF3 showed (*R*)-enoyl-coenzyme A (CoA) hydratase activity, suggesting that (*R*)-3-hydroxyacyl-CoA monomer units are supplied via the (*R*)-specific hydration of enoyl-CoA in *A. caviae*. The transconjugant of the *A. eutrophus* mutant expressing only $phaC_{Ac}$ effectively accumulated P(3HB-*co*-3HHx) up to 96 wt% of the cellular dry weight from octanoate in one-step cultivation.

The utilization of biological systems for production of biodegradable materials is becoming important as a solution of the problems concerning plastic waste and the global environment. Poly(3-hydroxyalkanoates) (PHA) are produced by a wide variety of bacteria as intracellular carbon- and energy-storage materials from renewable carbon resources, such as sugars or plant oils (2, 6, 18, 25). Since these bacterial PHA are biodegradable thermoplastics, they have attracted industrial attention as possible candidates for large-scale biotechnological products. At present, more than 90 different monomeric units have been found as constituents of PHA (37).

Bacterial PHA can be divided into two groups, depending on the number of carbon atoms in the monomeric units (35). One group of bacteria, including *Alcaligenes eutrophus*, produces short-chain-length PHA with C_3-to-C_5 monomer units, while the other group, including *Pseudomonas oleovorans*, synthesizes medium-chain-length PHA with C_6-to-C_{14} monomer units. Only a few reports are available for bacteria which can synthesize PHA consisting of both short- and medium-chain-length monomer units. For example, *Rhodospirillum rubrum* (3), *Rhodocyclus gelatinosus* (19), and *Rhodococcus ruber* (12) produce terpolymers consisting of C_4, C_5, and C_6 3-hydroxyalkanoate (3HA) units from hexanoate, and some pseudomonad strains accumulate PHA consisting of C_4-to-C_{12} 3HA units (16, 38). Our laboratory has found that a random copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx), P(3HB-*co*-3HHx), is produced by *Aeromonas caviae* FA440 isolated from soil (7, 32). This bacterium synthesizes the copolyester from alkanoic acids of even carbon numbers or from plant oils up to approximately 30 wt% of the cellular dry weight, with a 3HHx fraction ranging from 10 to 25 mol%. We have also demonstrated that P(3HB-*co*-3HHx) is a flexible material and that films of the copolymer show a high degree of elongation to break (<850%) (7).

PHA biosynthesis genes, including structural genes of PHA synthases, have been isolated and analyzed at a molecular level from various sources (18, 36). The genes of *A. eutrophus* are organized in a single operon as *phbC-A-B*, which are genes of PHA synthase, β-ketothiolase, and NADPH-acetoacetyl-coenzyme A (CoA) reductase, respectively (26, 31, 34). Whereas in *P. oleovorans*, two structural genes of PHA synthases (*phaC1* and *phaC2*) flanking a PHA depolymerase gene have been identified (14), PHA synthases of *Chromatium vinosum* (21) and *Thiocapsa pfennigii* (35) consist of two different types of subunits encoded by *phbC* and *phbE* in a single operon. Although cells of *T. pfennigii* accumulated only P(3HB) homopolymer from various carbon sources, a recombinant *Pseudomonas putida* strain harboring the PHA biosynthesis genes of *T. pfennigii* produced a new type of PHA consisting of 3HB, 3HHx, and 3-hydroxyoctanoate units from octanoate (20) or of PHA containing 4-hydroxy- and 5-hydroxyalkanoate units from the related carbon sources (40, 41). In this study, we cloned and sequenced the P(3HB-*co*-3HHx) biosynthesis genes of *A. caviae* FA440 to study the molecular organization. In addition, heterologous expression of the cloned genes was examined in PHA-negative mutants of *A. eutrophus* and *P. putida* to characterize the genes and the PHA-producing ability of the recombinant strains was investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *A. caviae*, *A. eutrophus*, and *P. putida* strains were cultivated at 30°C in a nutrient-rich medium containing 10 g of meat extract, 10 g of polypeptone, and 2 g of yeast extract in 1 liter of distilled water, and *Escherichia coli* strains were grown at 37°C on a Luria-Bertani medium (28). When needed, kanamycin (50 mg/liter) or ampicillin (50 mg/liter) was added to maintain the plasmids.

DNA manipulation. Isolation of total genomic DNA and plasmids, digestion of DNA with restriction endonucleases, and transformation of *E. coli* were carried out by standard procedures (28). Transconjugation of *A. eutrophus* or *P. putida*

* Corresponding author. Mailing address: Polymer Chemistry Laboratory, The Institute of Physical and Chemical Research (RIKEN), Hirosawa, Wako-shi, Saitama 351-01, Japan. Phone: 81-48-467-9402. Fax: 81-48-467-4662.

Downloaded from http://jb.asm.org/ on September 20, 2020 by guest

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
A. caviae FA440	Wild type	FERM P-3432 7, 32
A. eutrophus PHB$^-$4	PHA-negative mutant of H16	DSM 541, 30
P. putida GPp104	PHA-negative mutant of KT2442	14
E. coli		
DH5α	*deoR endA1 gyrA96 hsdR17* (r_K^- m_K^+) *recA1 relA1 supE44 thi-1* Δ(*lacZYA-argFV169*) ϕ80Δ*lacZ*ΔM15 F^- λ^-	Clontech
S17-1	*recA* and *tra* genes of plasmid RP4 integrated into the chromosome; auxotrophic for proline and thiamine	33
Plasmids		
pLA2917	Cosmid; Km^r Tc^r RK2 replicon Mob^+	1
pJRD215	Cosmid; Km^r Sm^r RSF1010 replicon Mob^+	4
pJRDEE50	pJRD215 derivative; $phaC_{Ac}$ ORF1 ORF3 ORF4 ORF5	This study
pJRDEE32	pJRD215 derivative; $phaC_{Ac}$ ORF1 ORF3	This study
pBluescriptII KS^+	Ap^r *lacPOZ* T7 and T3 promoter	Stratagene
pUC18	Ap^r *lacPOZ*	Takara
pEE32	pUC18 derivative; $phaC_{Ac}$ ORF1 ORF3	This study

with *E. coli* S17-1 harboring broad-host-range plasmids was performed as described by Friedrich et al. (8).

PCR. To amplify a partial fragment of the *A. caviae* PHA synthase gene from genomic DNA, we performed PCR with two primers [P1, 5′-CC(C/G)CC(C/G)TGGATCAA(T/C)AAGT(T/A)(T/C)TA(T/C)ATC-3′; P2, 5′-(G/C)AGCCA(G/C)GC(G/C)GTCCA(A/G)TC(G/C)GGCCACCA-3′] under 25 cycles of denaturation at 98°C for 20 s and annealing and elongation at 65°C for 2 min. The amplified DNA was purified by phenol extraction and ethanol precipitation.

Cloning of genes. Genomic DNA was partially digested with *Sau*3AI and ligated to the cosmid vector pLA2917 (1) linearized with *Bgl*II. The concatemeric ligation products were packaged by using Gigapack II (Stratagene), and the resultant phage particles were transfected to *E. coli* S17-1. The constructed cosmid library was screened by colony hybridization (28) with the PCR-amplified fragment as a probe to isolate the PHA biosynthesis genes of *A. caviae*. Preparation of the labeled probe and detection of the hybridization signals on membranes were carried out with a digoxigenin nucleic acid labeling and detection kit (Boehringer Mannheim).

DNA sequencing analysis. DNA fragments were subcloned into pBluescriptII KS^+ or pUC18, and nested sets of deletion clones were generated by using exonuclease III for DNA sequencing (28). DNA was sequenced in a DSQ-1000 DNA sequencer (Shimadzu Co., Kyoto, Japan) with a *Taq* cycle sequencing kit (Takara Co., Kyoto, Japan). Computer analysis of the resulting nucleotide sequence was performed with SDC-GENETYX genetic information processing software (Software Development Co., Tokyo, Japan).

Site-directed mutagenesis. To create restriction sites in the isolated genes, site-directed mutagenesis was carried out under the unique site elimination procedure developed by Deng and Nickoloff (5) with a U.S.E. mutagenesis kit (Pharmacia). Primers M1 and M2 (used for creation of *Bgl*II sites) and primers M3 and M4 (used for creation of *Bam*HI sites) were as follows: M1, 5′-GCCGATTGCCCCAGATCTACACTGTTCTGCC-3′; M2, 5′-GACGCTACGGGCTAGATCTCGCCTCGGGTGTG-3′; M3, 5′-CGCATGAGCGCAGGATCCCTGGAAGTAGGC-3′; and M4, 5′-GCCGTGACGGGGGGATCCGTGGTCAAGCTG-3′.

Production and analysis of PHA. One-step production of polyesters was carried out on a reciprocal shaker (130 strokes/min) at 30°C for 72 h in 500-ml flasks with 100 ml of a nitrogen-limited mineral salt medium, which was composed of 0.9 g of $Na_2HPO_4 \cdot 12H_2O$, 0.15 g of KH_2PO_4, 0.05 g of NH_4Cl, 0.02 g of $MgSO_4 \cdot 7H_2O$, and 0.1 ml of trace element solution (16). In the case of two-step production, cells were first cultivated in 100 ml of nutrient-rich medium for 12 h. Harvested and washed cells were then transferred into a nitrogen (NH_4Cl)-free mineral salt medium and incubated at 30°C for 48 h. Filter-sterilized carbon sources were added as indicated in the text. For maintenance of broad-host-range plasmids in *A. eutrophus* or *P. putida*, kanamycin was added to the medium at a concentration of 50 mg/liter. Cellular PHA content and composition were determined by gas chromatography after methanolysis of dried cells in the presence of 15% sulfuric acid, as described previously (16).

PHA synthase assay. Crude cell extracts of *A. eutrophus* transconjugants were prepared as described by Schubert et al. (31). The activity of PHA synthase was determined by spectroscopic assay according to the methods described by Valentin and Steinbüchel (43). (*R*)-3HB-CoA was synthesized by the mixed anhydride method described by Haywood et al. (11).

Enoyl-CoA hydratase assay. Recombinant *E. coli* cells were sonicated and centrifuged (20,000 × *g*, 20 min, 4°C), and the resulting soluble cell extracts were used as an enzyme solution. Enoyl-CoA hydratase activity was assayed by the hydration of crotonyl-CoA (Sigma) followed by measurement of the disappearance of absorbance at 263 nm derived from the decrease of the enoyl-thioester bond, as described by Moskowitz and Merrick (23). The configuration of 3HB-CoA produced by the hydratase was determined by coupling with the (*S*)-specific dehydrogenation. NAD^+ and (*S*)-3HA-CoA dehydrogenase (Sigma) were added after the hydration reaction had reached equilibrium, and the reduction of NAD^+ was monitored at 340 nm (23).

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. D88825.

RESULTS

Identification and cloning of the *A. caviae* PHA biosynthesis genes. For the identification of the PHA biosynthesis genes of *A. caviae*, a partial fragment of a PHA synthase gene was amplified from the genomic DNA and used as a specific probe. Two primers were designed from highly conserved regions among known PHA synthases (244-PPWINK(Y/F)YI-252 and 547-WWPDWTAWL-555; numbering corresponds to the *A. eutrophus* PHA synthase) (36), and PCR with the designed primers resulted in successful amplification of an approximately 900-bp fragment. Hybridization analysis gave only one positive signal for each of the *Eco*RI-, *Bam*HI-, *Hin*dIII-, and *Pst*I-digested genomic DNAs of *A. caviae* with a probe prepared from the amplified fragment. There were no detectable hybridization signals even under low-stringency conditions when the PHA synthase gene of *A. eutrophus* (26, 31, 34) was used as a probe. A cosmid library constructed in *E. coli* S17-1 was screened by colony hybridization, and one positive recombinant clone was isolated, which harbored a 20-kbp *A. caviae* genomic DNA fragment. A positive 11-kbp *Sal*I subfragment (SS110) was cloned into pBluescriptII KS^+, and further analysis showed that the PHA biosynthesis genes of *A. caviae* were located in a 5.0-kbp *Eco*RV-*Eco*RI subfragment, referred to as VE50.

Nucleotide sequence and structure of the PHA biosynthesis genes. A nucleotide sequence of the VE50 fragment was determined for both strands. Fig. 1b and 2 show the restriction map and the determined nucleotide sequence, respectively, of the VE50 fragment. Five open reading frames (ORFs) were identified (ORF1 to -5) in the fragment by computer analysis, as shown in Fig. 1c.

ORF2 (1,782 bp), which is the largest gene in this fragment, encoded a protein composed of 594 amino acids with a mo-

Downloaded from http://jb.asm.org/ on September 20, 2020 by guest

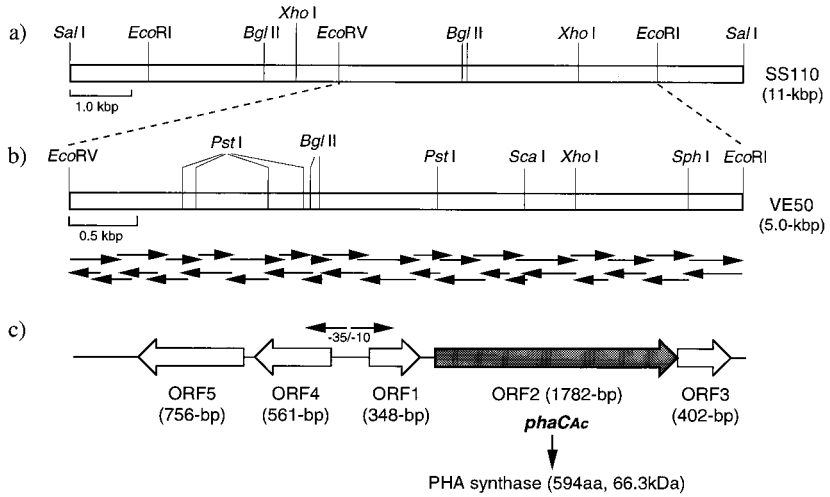

FIG. 1. Organization of *A. caviae* PHA biosynthesis genes, restriction endonuclease sites, and DNA sequencing strategy. (a) Restriction map of SS110 fragment. (b) Restriction map and sequencing strategy of VE50 subfragment. Arrows indicate sequence strategy. (c) Organization of $phaC_{Ac}$, ORF1, ORF3, ORF4, and ORF5. aa, amino acids.

lecular mass of 66,334 Da. Figure 3 shows a partial alignment and the identities of its deduced amino acid sequence with known PHA synthases from 11 microorganisms. Relatively high identities were obtained with the synthases of *Acinetobacter* sp. (45.1%) (29) and *A. eutrophus* (42.7%); therefore, ORF2 was concluded to represent a structural gene of *A. caviae* PHA synthase, and it was referred to as $phaC_{Ac}$. The calculated molecular mass of the translated PHA synthase was almost consistent with that of *A. eutrophus* (63,940 Da) and those of *P. oleovorans* (62,400 and 62,600 Da) (14). The propagation of a polyester chain has been proposed to include the formation of an acyl-*S* enzyme intermediate at two thiol groups and the transesterification to a propagating chain (17). Cys-319 in the *A. eutrophus* synthase has been demonstrated by mutagenic analysis to play important roles in the catalytic cycles (9), and the corresponding Cys residue is conserved in the PHA synthase of *A. caviae* at the same position, 319, in a lipase box-like sequence. Another active site has been proposed to be a thiol group of a 4′-phosphopantethein moiety which post-translationally modifies the synthase of *A. eutrophus* in *E. coli* (9). A candidate for the modified residue, Ser-260, is also found in the amino acid sequence deduced from $phaC_{Ac}$.

ORF1 (348 bp) and ORF3 (402 bp) were located in upstream and downstream regions, respectively, of $phaC_{Ac}$, and ORF4 (561 bp) and ORF5 (756 bp) were oriented in the opposite direction to the other three genes. Several −35 to −10 consensus sequences of a σ^{70}-dependent promoter (10) were found between ORF1 and ORF4 on both strands, suggesting that the approximately 300-bp region is a putative promoter region for these five genes. The ATG start codon of ORF3 overlapped with the TGA stop codon of $phaC_{Ac}$, and an inverted repeat, which may serve as a transcriptional termination signal, was identified in the downstream region of ORF3 (nucleotides 4899 to 4930) with the structural free energy of −166 kJ/mol. The presence of ribosome binding sequences 5 to 8 bp upstream of the ATG start codon of all genes suggests the translation of these five genes.

Complementation studies and heterologous expression. To confirm whether the cloned fragments harbor functionally active PHA biosynthesis genes, heterologous expression of the genes was investigated in the PHA-negative mutants PHB$^-$4 of *A. eutrophus* (30) and GPp104 of *P. putida* (14). The VE50 fragment (containing $phaC_{Ac}$, ORF1, ORF3, ORF4, and ORF5) and a 3.2-kbp *Bgl*II-*Eco*RI fragment (containing $phaC_{Ac}$, ORF1, and ORF3), both of which harbored the putative promoter region, were converted to *Eco*RI restriction fragments referred to as EE50 and EE32, respectively, with a p*Eco*RI linker. These two fragments were inserted into a broad-host-range vector, pJRD215 (4), at the unique *Eco*RI site, and the resultant plasmids, pJRDEE50 and pJRDEE32, were mobilized from *E. coli* S17-1 to *A. eutrophus* PHB$^-$4 or to *P. putida* GPp104. The transconjugants were cultivated in a mineral salt medium to promote the PHA biosynthesis from sugar (fructose for transconjugants of PHB$^-$4 or gluconate for those of GPp104), hexanoate, or octanoate as a carbon source.

Table 2 shows the results of PHA accumulation in the recombinant strains by one-step cultivation. The plasmids pJRDEE50 and pJRDEE32 not only could complement the deficiency of PHA synthase in the mutant strains but also could confer the ability to synthesize P(3HB-*co*-3HHx) copolymer on the hosts. *A. eutrophus* PHB$^-$4 harboring pJRDEE32 produced P(3HB) homopolymer from fructose (53 wt% of the cellular dry weight) and P(3HB-*co*-3HHx) copolymer, with 22 mol% of the 3HHx fraction from octanoate (33 wt%), while the strain harboring pJRDEE50 accumulated only a small amount of polyesters (0 to 7 wt%). *P. putida* GPp104 harboring these plasmids accumulated more than 40 wt% of P(3HB-*co*-3HHx) from hexanoate or octanoate, with a high 3HHx composition. The mole fraction of the 3HHx unit reached 40 mol% in the hexanoate-grown cells. These strains synthesized P(3HB-*co*-3HHx) not only from the carboxylic acids but also from gluconate, although the content was low (4 wt%).

Furthermore, ORF1 and/or ORF3 were deleted from the EE32 fragment, and the accumulation of PHA in transconjugants of PHB$^-$4 harboring the deleted clones was investigated. The EE32 fragment was ligated to pUC18 to form a recombinant plasmid called pEE32. Two *Bgl*II sites across the coding region of ORF1 or two *Bam*HI sites across that of ORF3 were created by site-directed mutagenesis, and elimination of the *Bgl*II or *Bam*HI fragment from the modified plasmids gave pEE32d1 and pEE32d3, respectively. pEE32d13 was also constructed by eliminating both the *Bgl*II and *Bam*HI fragments. The deleted *Eco*RI restriction fragments of these plasmids were inserted into pJRD215, resulting in the formation of

Downloaded from http://jb.asm.org/ on September 20, 2020 by guest

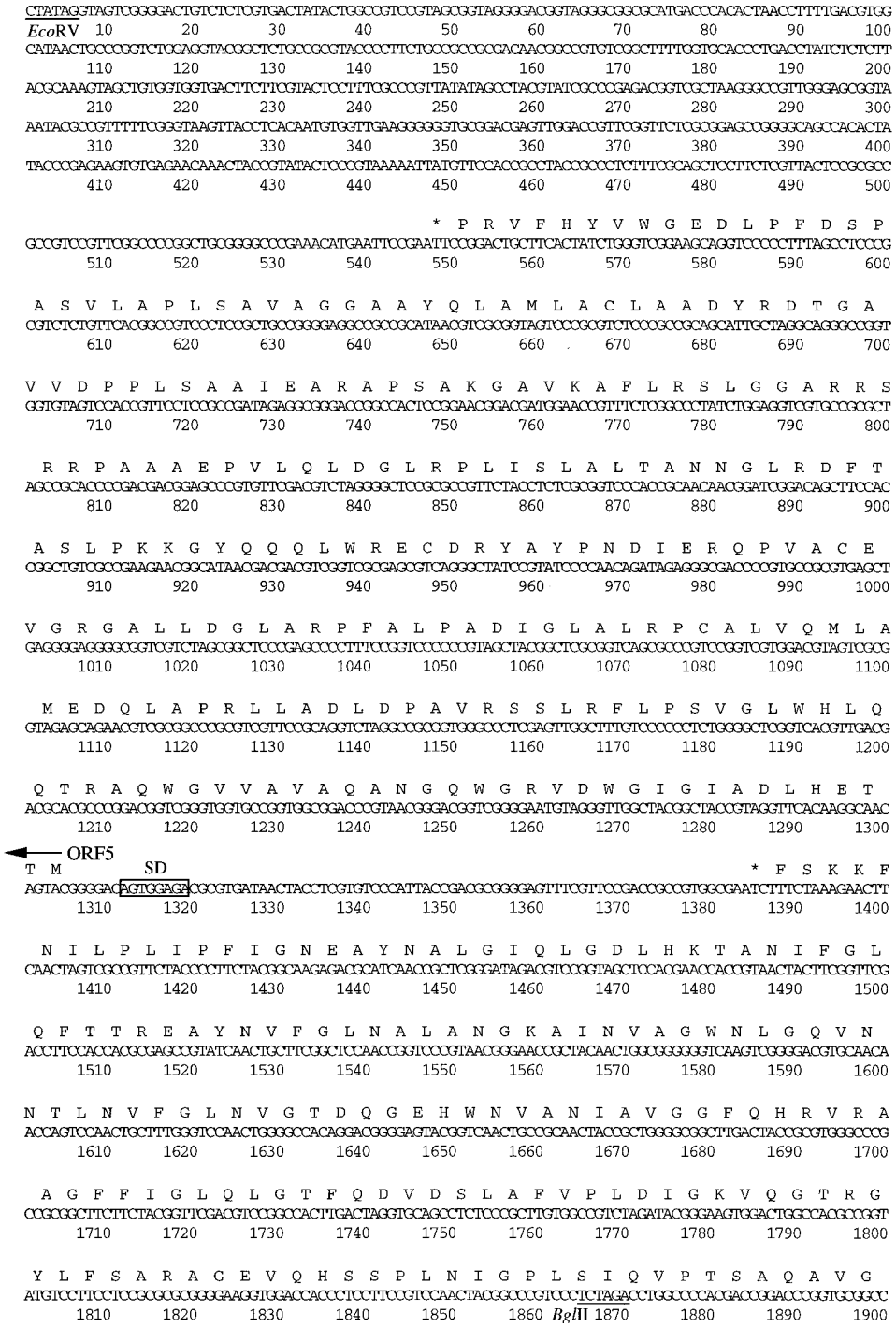

FIG. 2. Nucleotide sequence of a 5,051-bp region containing the *phaC*$_{Ac}$ gene along with the deduced amino acid sequence. The putative ribosome binding sequences are boxed and indicated by SD. The −10 and −35 regions of the σ^{70}-dependent promoter are labeled. A potential stem-loop structure is shown by the facing arrows. P1 and P2 indicate the positions of the primers used for PCR. M1, M2, M3, and M4 indicate the restriction sites created by site-directed mutagenesis. Other restriction sites are underlined and labeled. Stop codons are indicated by asterisks.

pJRDEE32d1, harboring *phaC*$_{Ac}$ and ORF3, pJRDEE32d3, harboring *phaC*$_{Ac}$ and ORF1, and pJRDEE32d13, harboring only *phaC*$_{Ac}$. *A. eutrophus* PHB^{-}4 was transformed by conjugational transfer of these recombinant plasmids, and the transconjugants were cultivated in a nitrogen-limited mineral salt medium. As shown in Table 3, all the transconjugants of PHB^{-}4 accumulated P(3HB) homopolymer from fructose, and the deletion of ORF1 and/or ORF3 slightly increased the PHA content in the cells. When the recombinant PHB^{-}4 strains were grown on octanoate or hexanoate, the content of P(3HB-*co*-3HHx) copolymer was remarkably increased by the deletion of ORFs. Especially on octanoate, the PHA content

Downloaded from http://jb.asm.org/ on September 20, 2020 by guest

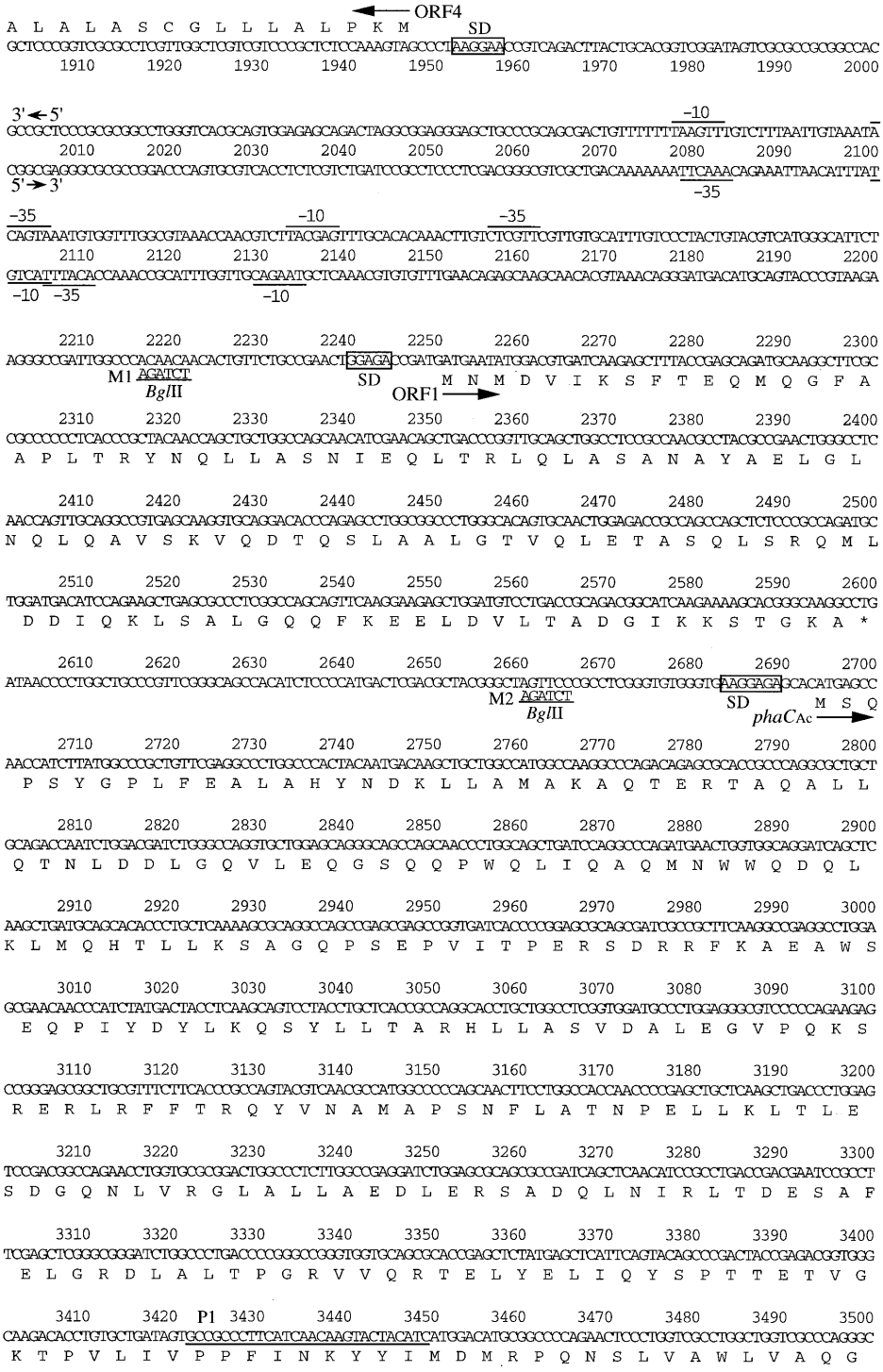

FIG. 2—*Continued.*

reached 92% of the cell weight (dry wt) after deletion of ORF1 or ORF3 from the EE32 fragment, and 96% (dry wt) after deletion of both genes. A similar tendency was also observed in the case of two-step cultivation.

Table 4 shows PHA synthase activities in the transconjugants of PHB$^-$4 at the early stationary growth phase. When octanoate was fed as a sole carbon source, the PHA synthase activity in the cells expressing *phaC*$_{Ac}$ with ORF1 (pJRDEE32d3) was fivefold higher than that in the cells coexpressing the three genes (pJRDEE32). Furthermore, the strain expressing only *phaC*$_{Ac}$ without both ORF1 and ORF3 (pJRDEE32d13) showed 38-fold higher activity than that harboring pJRDEE32. In contrast, the transconjugant harboring pJRDEE32d1 showed lower activities in spite of the high PHA content than did those harboring pJRDEE32 or pJRDEE32d3. The three strains harboring these deleted plasmids accumulated P(3HB-*co*-3HHx)

Downloaded from http://jb.asm.org/ on September 20, 2020 by guest

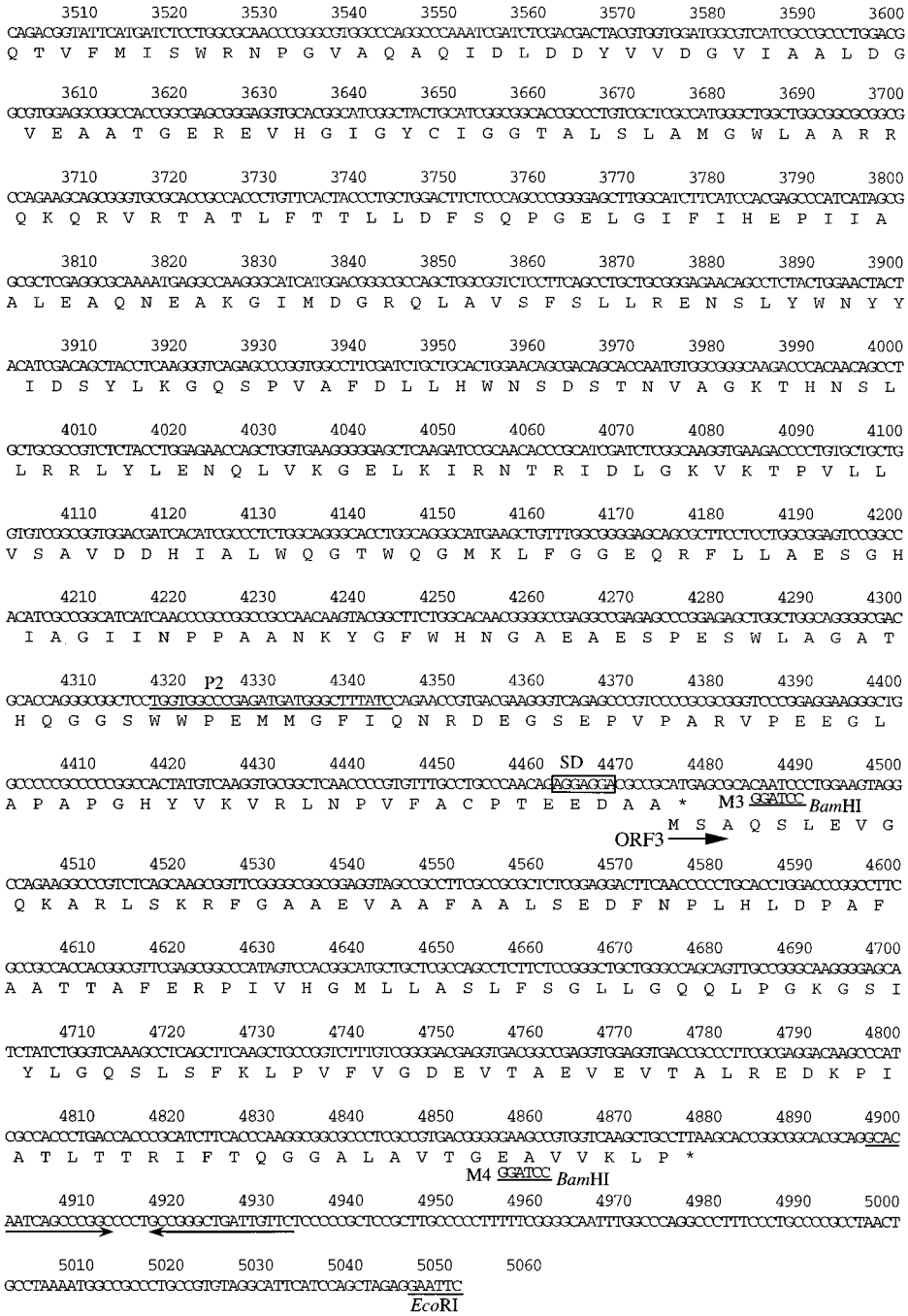

FIG. 2—*Continued.*

up to more than 92 wt% from octanoate after 72 h cultivation, but they showed quite different synthase activities. These results suggested that the PHA synthase activity does not relate directly to the PHA content accumulated in cells.

The deletion of ORFs from the EE32 fragment did not seriously affect the composition of copolyesters accumulated in octanoate-grown cells (ranging from 12 to 22 mol% of the 3HHx fraction). However, it is of interest to note that the mole fractions of the 3HHx unit in copolyesters synthesized from hexanoate were increased by the deletion of ORF3. P(3HB-*co*-3HHx), consisting of almost equimolar amounts of 3HB and 3HHx units, could be obtained by two-step cultivation of the strains harboring pJRDEE32d3 or pJRDEE32d13.

Enoyl-CoA hydratase assay. No significant identities of the amino acid sequences deduced from the nucleotide sequences of identified ORFs were detected with those of primary structures of any proteins in databases, except for the translated product of ORF3, which showed a partial identity with a putative enoyl-CoA hydratase domain of *Saccharomyces cerevisiae* β-oxidation multifunctional protein (38.4% of 73 amino acids) (13). The ORF3 product also exhibited a weak identity with *Clostridium difficile* crotonase (22.8% of 114 amino acids)

Downloaded from http://jb.asm.org/ on September 20, 2020 by guest

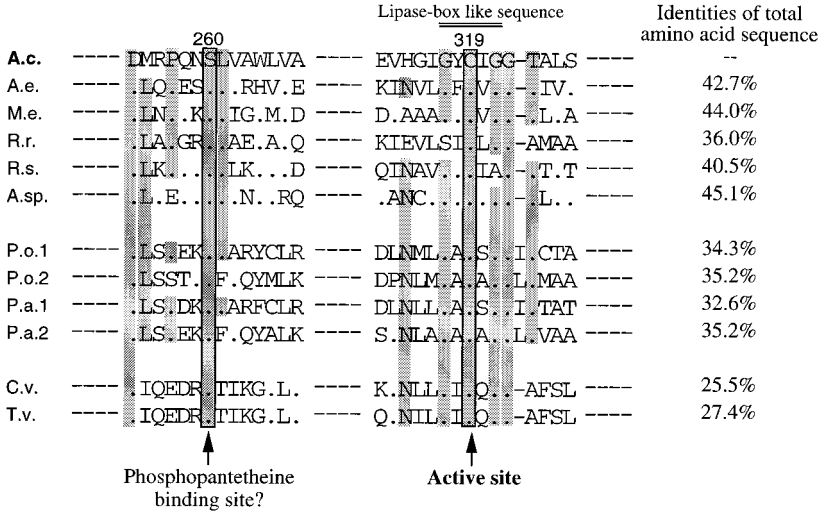

FIG. 3. Partial alignment and identities of the deduced amino acid sequence of the PHA synthase from *A. caviae* with those from *A. eutrophus* (A.e.) (26, 31, 34), *Methylobacterium extorquens* (M.e.) (42), *R. ruber* (R.r.) (27), *R. sphaeroides* (R.s.) (15), *Acinetobacter* sp. (A.sp.) (29), *P. oleovorans* (P.o.1 and P.o.2) (14), *P. aeruginosa* (P.a.1 and P.a.2) (39), *C. vinosum* (C.v.) (21), and *Thiocystis violacea* (T.v.) (22). Dots, amino acids identical to the *A. caviae* sequence; shading, amino acids which are identical in at least eight PHA synthases.

(24). Therefore, soluble extracts of recombinant *E. coli* harboring the PHA biosynthetic genes of *A. caviae* were prepared, and enoyl-CoA hydratase activity was assayed with crotonyl-CoA as a substrate. The results are given in Table 5. *E. coli* harboring pEE32 or pEE32d1 exhibited a high enoyl-CoA hydratase activity, while the activity in the cells harboring pEE32d3 or pEE32d13 was as low as that in a control strain harboring pUC18. To investigate the stereospecificity of the hydration reaction, NAD^+ and (*S*)-3HA-CoA dehydrogenase were added to the reaction mixture after the hydration of crotonyl-CoA had reached equilibrium (23), but the formation of NADH linked with the oxidation of (*S*)-3HB-CoA was not observed. These results strongly suggest that ORF3 is a structural gene of (*R*)-specific enoyl-CoA hydratase. The β-oxidation multifunctional protein of *S. cerevisiae* encoded by the *fox2* gene has been reported to catalyze the hydration of enoyl-CoA with (*R*)-specificity (13). The evolutionary relationship between *A. caviae* ORF3 and *S. cerevisiae fox2* may be an interesting subject.

DISCUSSION

There are only a few studies on bacteria capable of incorporating both short- and medium-chain-length 3HA units into polyester chains. *R. ruber* (12) and *R. rubrum* (3) are known to synthesize PHA with C_4-to-C_6 3HA units, and the PHA synthase genes of these bacteria have been cloned (15, 27). However, the substrate specificities of the translated PHA synthases were not investigated thoroughly. In this study, cloning of PHA biosynthesis genes, including a structural gene of PHA synthase from *A. caviae* (*phaC*$_{Ac}$), was performed together with an investigation of the PHA-producing ability of recombinant strains harboring the cloned genes.

A partial fragment of a PHA synthase gene was successfully amplified from genomic DNA of *A. caviae* by PCR with primers designed from a highly conserved region among various PHA synthases. The amplified fragment was then used as a specific probe for identification and isolation of the PHA biosynthesis genes of *A. caviae*. These PCR primers are expected to be useful for cloning of PHA synthase genes from other bacteria.

The nucleotide sequence indicated that *phaC*$_{Ac}$ was clustered with four ORFs (ORF1, -3, -4, and -5) and one putative promoter region in a 5.0-kbp genomic fragment. The deduced amino acid sequence of *A. caviae* PHA synthase shows 42.7% identity with the synthase of *A. eutrophus* (specific for short-chain-length 3HA), which is higher than its 32.6 to 35.2% identity with those of pseudomonads (specific for medium- and

TABLE 2. Accumulation of PHA in recombinant strains harboring PHA biosynthesis genes of *A. caviae*[a]

Strain	Plasmid	Carbon source	PHA content (wt%)	Composition (mol%)	
				3HB	3HHx
A. eutrophus PHB$^-$4	pJRD215	Fructose	0		
		Hexanoate	0		
		Octanoate	0		
	pJRDEE50	Fructose	7	100	0
		Hexanoate	Trace		
		Octanoate	6	96	4
	pJRDEE32	Fructose	53	100	0
		Hexanoate	6	83	17
		Octanoate	33	78	22
P. putida GPp104	pJRD215	Gluconate	0		
		Hexanoate	0		
		Octanoate	0		
	pJRDEE50	Gluconate	4	71	29
		Hexanoate	42	61	39
		Octanoate	41	71	29
	pJRDEE32	Gluconate	4	71	29
		Hexanoate	38	60	40
		Octanoate	48	69	31

[a] Cells were cultivated in a nitrogen-limited mineral salt medium containing fructose (0.5% wt/vol), the sodium salts of gluconate (1.5% wt/vol), and octanoate or hexanoate (0.1% wt/vol × 5 for *A. eutrophus* strains or 0.5% wt/vol for *P. putida* strains) as a sole carbon source for 72 h at 30°C.

Downloaded from http://jb.asm.org/ on September 20, 2020 by guest

TABLE 3. Accumulation of PHA in recombinant strains of *A. eutrophus* PHB$^-$4 harboring deleted clones of PHA biosynthesis genes of *A. caviae*

Plasmid (relevant markers)	Carbon source	One-step cultivation[a]			Two-step cultivation[b]		
		PHA content (wt%)	Composition (mol%)		PHA content (wt%)	Composition (mol%)	
			3HB	3HH		3HB	3HHx
pJRDEE32 (*phaC$_{Ac}$*, ORF1, ORF3)	Fructose	53	100	0			
	Hexanoate	6	83	17	4	76	24
	Octanoate	33	78	22	6	85	15
pJRDEE32d1 (*phaC$_{Ac}$*, ORF3)	Fructose	66	100	0			
	Hexanoate	78	84	16	25	77	23
	Octanoate	92	87	13	53	85	14
pJRDEE32d3 (*phaC$_{Ac}$*, ORF1)	Fructose	66	100	0			
	Hexanoate	44	75	25	19	53	47
	Octanoate	92	88	12	23	87	13
pJRDEE32d13 (*phaC$_{Ac}$*)	Fructose	73	100	0			
	Hexanoate	72	72	28	26	50	50
	Octanoate	96	85	15	50	80	20

[a] Cells were cultivated in a nitrogen-limited mineral salt medium containing fructose (0.5% wt/vol) and sodium hexanoate or sodium octanoate (0.1% wt/vol × 5) as a sole carbon source for 72 h at 30°C.
[b] Cells were grown in a nutrient-rich medium for 12 h at 30°C, transferred into a nitrogen-free mineral salt medium containing sodium hexanoate or sodium octanoate (0.25% wt/vol × 2) as a carbon source, and incubated for 48 h at 30°C.

long-chain-length 3HA). Although *R. ruber* synthesizes copolyesters of C_4 to C_6 units similar to those of *A. caviae* (12), the PHA synthase of *R. ruber* has closer identity to the synthases of *Pseudomonas aeruginosa* than to those of *A. eutrophus* and *A. caviae*. It is difficult to predict the substrate specificity of PHA synthases on the basis of their primary structures.

Heterologous expression of *phaC$_{Ac}$* in the PHA-negative mutants PHB$^-$4 of *A. eutrophus* and GPp104 of *P. putida* resulted in the accumulation of P(3HB-*co*-3HHx) copolyesters from hexanoate or octanoate and of P(3HB-*co*-3-hydroxyvalerate) from pentanoate (data not shown). A 3-hydroxyoctanoate unit was never detected, even in the copolyesters produced by transconjugants of GPp104 from octanoate. These results indicate that the *A. caviae* PHA synthase is active toward C_4-to-C_6 3HA-CoA and that the composition of the copolyester produced by *A. caviae* FA440 reflects the substrate specificity of the PHA synthase. The acceptance of 3HHx-CoA as a substrate is a significant difference between the *A. caviae* PHA synthase and other synthases, being specific for short-chain-length C_3-to-C_5 3HA-CoA only.

E. coli strains expressing the ORF3 gene of *A. caviae* showed (*R*)-specific enoyl-CoA hydratase activity, suggesting that the translated product of ORF3 functions as an enzyme in a monomer-supplying pathway for PHA-biosynthesis. Figure 4 shows a proposed PHA biosynthesis pathway in *A. caviae*. Acyl-CoA derived from alkanoic acids or oils is degraded via cyclic β-oxidation, resulting in the formation of enoyl-CoA intermediates of different chain lengths. These intermediates may be converted to (*R*)-3HA-CoA by the (*R*)-specific enoyl-CoA hydratase encoded by ORF3, and the resultant (*R*)-3HA-CoA of 4 to 6 carbon atoms may be incorporated into a growing polyester chain by the function of PHA synthase. Hence, P(3HB-*co*-3HHx) is synthesized from alkanoic acids of even carbon numbers or from oils. Small amounts (5 mol% ± 2 mol%) of

TABLE 4. PHA synthase activity and PHA accumulation in recombinant strains of *A. eutrophus* at the early stationary growth phase[a]

Strain	Plasmid (relevant markers)	Carbon source	PHA synthase[b] (U/g of protein)	PHA content (wt%)	Composition (mol%)	
					3HB	3HHx
H16	pJRD215	Fructose	138	60	100	0
		Octanoate	61	67	100	0
PHB$^-$4	pJRD215	Fructose	5	0		
		Octanoate	3	0		
PHB$^-$4	pJRDEE32 (*phaC$_{Ac}$*, ORF1, ORF3)	Fructose	145	25	100	0
		Octanoate	20	12	61	39
PHB$^-$4	pJRDEE32d1 (*phaC$_{Ac}$*, ORF3)	Fructose	33	49	100	0
		Octanoate	13	51	88	12
PHB$^-$4	pJRDEE32d3 (*phaC$_{Ac}$*, ORF1)	Fructose	110	28	100	0
		Octanoate	114	37	85	15
PHB$^-$4	pJRDEE32d13 (*phaC$_{Ac}$*)	Fructose	425	69	100	0
		Octanoate	770	69	85	25

[a] Cells were cultivated in a nitrogen-limited mineral salt medium containing fructose (0.5% wt/vol) or sodium octanoate (0.1% wt/vol × 3) as a sole carbon source for 30 h at 30°C.
[b] PHA synthesis activity for (*R*)-3HB-CoA.

Downloaded from http://jb.asm.org/ on September 20, 2020 by guest

TABLE 5. Enoyl-CoA hydratase activity in recombinant strains of *E. coli* DH5α[a]

Plasmid	Relevant markers	Enoyl-CoA hydratase[b] (U/g of protein)
pUC18		39
pEE32	$phaC_{Ac}$, ORF1, ORF3	617
pEE32d1	$phaC_{Ac}$, ORF3	323
pEE32d3	$phaC_{Ac}$, ORF1	31
pEE32d13	$phaC_{Ac}$	46

[a] Cells were cultivated in a Luria-Bertani medium for 24 h at 37°C.
[b] Hydration activity for crotonyl-CoA.

a 3HB unit were reported to be incorporated into a copolyester with a 3-hydroxyvalerate unit (95 mol% ± 2 mol%) by *A. caviae* from alkanoic acids of odd carbon numbers (7). In *A. caviae*, there are low activities of β-ketothiolase (0.09 U/mg of protein) and NADH-acetoacetyl-CoA dehydrogenase (0.06 U/mg of protein), but no activity of NADPH-acetoacetyl-CoA reductase was detected. The small amount of a 3HB unit in the copolyesters may be supplied from two acetyl-CoA molecules via four-step reactions catalyzed by β-ketothiolase, NADH-acetoacetyl-CoA dehydrogenase, crotonase [(*S*)-specific enoyl-CoA hydratase], and (*R*)-specific enoyl-CoA hydratase (Fig. 4), which is similar to the pathway in *R. rubrum* (23). The deletion of ORF3 from the EE32 fragment resulted in an enrichment of the 3HHx fraction in P(3HB-*co*-3HHx) produced by the recombinant strains of *A. eutrophus* PHB^{-}4 (Table 3). This result may suggest the major contribution of the ORF3 product in supplying a 3HB unit; that is, the (*R*)-specific enoyl-CoA hydratase encoded by ORF3 may be more specific for the hydration of crotonyl-CoA than for that of medium-chain-length enoyl-CoA.

The transconjugant of *A. eutrophus* PHB^{-}4 harboring pJRDEE50 accumulated a smaller amount of polyesters than that harboring pJRDEE32, while there was little difference in the PHA content and composition between transconjugants of

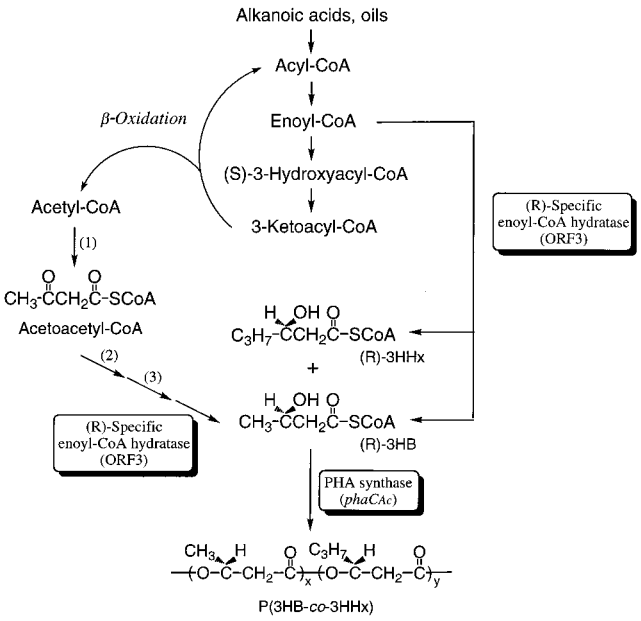

FIG. 4. Proposed pathway of P(3HB-*co*-3HHx)-biosynthesis in *A. caviae* from alkanoic acids or oils. (1) β-Ketothiolase; (2) NADH-acetoacetyl-CoA dehydrogenase; (3) crotonase [(*S*)-specific enoyl-CoA hydratase].

P. putida GPp104 harboring pJRDEE50 and those harboring pJRDEE32. The functions of ORF4 and ORF5 in PHA biosynthesis are still unknown. The deletion of ORF1 and/or ORF3 from the EE32 fragment drastically increased the P(3HB-*co*-3HHx) content in the cells of recombinant PHB^{-}4 when hexanoate or octanoate was fed as a sole carbon source. Here, it is noted that a PHA content of 96 wt% was achieved in the strain harboring pJRDEE32d13 from octanoate. The coexpression of ORF1 and ORF3 with $phaC_{Ac}$ seems to lead to a decrease in intracellular PHA content. Enzymatic analysis revealed that the deletion of the surrounding genes also affected the activity of PHA synthase. However, the PHA content accumulated in cells was not correlated to the level of PHA synthase activity (Table 4). For example, the PHA content in octanoate-grown cells harboring pJRDEE32d13 was only slightly higher than that of cells harboring pJRDEE32d1 at both the early stationary (30 h) and late stationary (72 h) phases, even though the former strain showed 60-fold higher synthase activity than the latter. In addition, the effects of the deletion of the ORF3 gene on the activity of PHA synthase and on the cellular content of PHA cannot be explained by the catalytic function of the translated product, (*R*)-specific enoyl-CoA hydratase. Further studies, including the investigation of transcriptional efficiency of each transconjugant, will be necessary to elucidate the characters and functions of these ORFs.

Liebergesell et al. (20) have reported that PHA biosynthesis genes of the anoxygenic phototrophic bacteria *C. vinosum*, *Rhodobacter sphaeroides*, and *T. pfennigii* conferred the ability to synthesize P(3HB-*co*-3HHx) from octanoate on *P. putida* GPp104. It is interesting that the PHA synthases of *C. vinosum* and *T. pfennigii* exhibited similar specificity to that of *A. caviae* in spite of their quite different structures. They have also reported that P(3HB) homopolymer was accumulated in the octanoate-grown cells of *A. eutrophus* PHB^{-}4 harboring the genes of *T. pfennigii*, in contrast to the production of PHA containing nearly a half-molar ratio of the 3HHx fraction by the GPp104 strain expressing the same genes. In this study, the transconjugant of PHB^{-}4 harboring pJRDEE32d13, expressing only $phaC_{Ac}$ as a foreign gene, efficiently accumulated P(3HB-*co*-3HHx) from hexanoate or octanoate, and the 3HHx fraction in the copolyester reached 50 mol% in the recombinant cells from hexanoate by two-step cultivation. Apparently, *A. eutrophus* cells have the ability to supply the (*R*)-3HHx-CoA thioester intermediate from these carboxylic acids. The P(3HB) accumulation from octanoate in recombinant PHB^{-}4 strains described by Liebergesell et al. may be caused by the effects of additional genes in the cloned fragment which were introduced into the host together with the PHA synthase gene.

In conclusion, this study not only gives information on PHA biosynthesis genes of *A. caviae* but also demonstrates the usefulness of the recombination techniques for an efficient production of copolyesters consisting of short- and medium-chain-length 3HA-CoA. Further studies will be done to clarify the mechanism of synthesis and accumulation of PHA in *A. caviae* and in recombinant strains harboring the PHA biosynthesis genes of *A. caviae.*

ACKNOWLEDGMENTS

We are indebted to H. G. Schlegel (Georg-August-Universität) for the kind gifts of *A. eutrophus* PHB^{-}4 and *E. coli* S17-1 and to B. Witholt (ETH) for those of *P. putida* GPp104 and plasmid pJRD215 used in this work.

REFERENCES

1. **Allen, L. N., and R. S. Hanson.** 1985. Construction of broad-host-range cosmid cloning vectors: identification of genes necessary for growth of *Methy-*

Downloaded from http://jb.asm.org/ on September 20, 2020 by guest

lobacterium organophilum on methanol. J. Bacteriol. **161:**955–962.
2. **Anderson, A. J., and E. A. Dawes.** 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. **54:**450–472.
3. **Brandl, H., E. J. Knee, R. C. Fuller, R. A. Gross, and R. W. Renz.** 1989. Ability of the phototrophic bacterium *Rhodospirillum rubrum* to produce various poly(β-hydroxyalkanoates): potential source for biodegradable polyesters. Int. J. Biol. Macromol. **11:**49–55.
4. **Davison, J., M. Heusterspreute, N. Chevalier, V. Ha-Thi, and F. Brunel.** 1987. Vectors with restriction site banks. pJRD215, a wide-host-range cosmid vector with multiple cloning sites. Gene **51:**275–280.
5. **Deng, W. P., and J. A. Nickoloff.** 1992. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. Anal. Biochem. **200:**81–88.
6. **Doi, Y.** 1990. Microbial polyesters. VCH Publishers, New York, N.Y.
7. **Doi, Y., S. Kitamura, and H. Abe.** 1995. Microbial synthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). Macromolecules **28:**4822–4828.
8. **Friedrich, B., C. Hogrefe, and H. G. Schlegel.** 1981. Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J. Bacteriol. **147:**198–205.
9. **Gerngross, T. U., K. D. Snell, O. P. Peoples, A. J. Sinskey, E. Csuhai, S. Masamune, and J. Stubbe.** 1994. Overexpression and purification of the soluble polyhydroxyalkanoate synthase from *Alcaligenes eutrophus*. Evidence for a required posttranslational modification for catalytic activity. Biochemistry **33:**9311–9320.
10. **Hawrey, D. K., and W. R. McClure.** 1983. Composition and analysis of *Escherichia coli* promoter sequences. Nucleic Acids Res. **11:**2237–2255.
11. **Haywood, G. W., A. J. Anderson, and E. A. Dawes.** 1989. The importance of PHB-synthase substrate specificity in polyhydroxyalkanoate synthesis by *Alcaligenes eutrophus*. FEMS Microbiol. Lett. **57:**1–6.
12. **Haywood, G. W., A. J. Anderson, G. A. Williams, E. A. Dawes, and D. F. Ewing.** 1991. Accumulation of a poly(hydroxyalkanoate) copolymer containing primarily 3-hydroxyvalerate from simple carbohydrate substrates by *Rhodococcus* sp. NICMB 40126. Int. J. Biol. Macromol. **13:**83–87.
13. **Hiltunen, J. K., B. Wenzel, A. Beyer, R. Erdmann, A. Fosså, and W. H. Kunau.** 1992. Peroxisomal multifunctional β-oxidation protein of *Saccharomyces cerevisiae*. J. Biol. Chem. **267:**6646–6653.
14. **Huisman, G. W., E. W. Wonink, R. Meima, B. Kazemier, P. Terpstra, and B. Witholt.** 1991. Metabolism of poly(3-hydroxyalkanoates) (PHAs) by *Pseudomonas oleovorans*. J. Biol. Chem. **266:**2191–2198.
15. **Hustede, E., and A. Steinbüchel.** 1993. Characterization of the polyhydroxyalkanoate synthase gene locus of *Rhodobacter sphaeroides*. Biotechnol. Lett. **15:**709–714.
16. **Kato, M., H. J. Bao, C. K. Kang, T. Fukui, and Y. Doi.** 1996. Production of a novel copolyester of 3-hydroxybutyric acids and medium-chain-length 3-hydroxyalkanoic acids by *Pseudomonas* sp. 61-3 from sugars. Appl. Microbiol. Biotechnol. **45:**363–370.
17. **Kawaguchi, Y., and Y. Doi.** 1992. Kinetics and mechanism of synthesis and degradation of poly(3-hydroxybutyrate) in *Alcaligenes eutrophus*. Macromolecules **25:**2324–2329.
18. **Lee, S. Y.** 1996. Bacterial polyhydroxyalkanoates. Biotechnol. Bioeng. **49:**1–14.
19. **Liebergesell, M., E. Hustede, A. Timm, A. Steinbüchel, R. C. Fuller, R. W. Lenz, and H. G. Schlegel.** 1991. Formation of poly(3-hydroxyalkanoates) by phototrophic and chemolithotrophic bacteria. Arch. Microbiol. **155:**415–421.
20. **Liebergesell, M., F. Mayer, and A. Steinbüchel.** 1993. Analysis of polyhydroxyalkanoic acid-biosynthetic genes of anoxygenic phototrophic bacteria reveals synthesis of a polyester exhibiting an unusual composition. Appl. Microbiol. Biotechnol. **40:**292–300.
21. **Liebergesell, M., and A. Steinbüchel.** 1992. Cloning and nucleotide sequences of genes relevant for biosynthesis of poly(3-hydroxybutyric acid) in *Chromatium vinosum* strain D. Eur. J. Biochem. **209:**135–150.
22. **Liebergesell, M., and A. Steinbüchel.** 1993. Cloning and molecular analysis of the poly(3-hydroxybutyric acid) biosynthetic genes of *Thiocystis violacea*. Appl. Microbiol. Biotechnol. **38:**493–501.
23. **Moskowitz, G. J., and J. M. Merrick.** 1969. Metabolism of poly-β-hydroxybutyrate. Enzymatic synthesis of D-(-)-β-hydroxybutyryl coenzyme A by an enoyl hydrase from *Rhodospirillum rubrum*. Biochemistry **8:**2748–2755.
24. **Mullany, P., C. L. Clayton, M. J. Pallen, R. Slone, A. al-Saleh, and S. Tabaqchali.** 1994. Genes encoding homologues of three consecutive enzymes in the butyrate/butanol-producing pathway of *Clostridium acetobutylicum* are clustered on the *Clostridium difficile* chromosome. FEMS Microbiol. Lett. **124:**61–67.
25. **Müller, H. M., and D. Seebach.** 1993. Poly(hydroxyalkanoates): a fifth class of physiologically important organic biopolymers? Angew. Chem. Int. Ed. Engl. **32:**477–502.
26. **Peoples, O. P., and A. J. Sinskey.** 1989. Poly-β-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). J. Biol. Chem. **264:**15298–15303.
27. **Pieper, U., and A. Steinbüchel.** 1992. Identification, cloning and sequence analysis of the poly(3-hydroxyalkanoic acid) synthase gene of the gram-positive bacterium *Rhodococcus ruber*. FEMS Microbiol. Lett. **96:**73–80.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Schembri, M. A., R. C. Bayly, and K. Davies.** 1994. Cloning and analysis of the polyhydroxyalkanoic acid synthase gene from *Acinetobacter* sp. Evidence that the gene is both plasmid and chromosomally located. FEMS Microbiol. Lett. **118:**145–152.
30. **Schlegel, H. G., R. Lafferty, and I. Krauss.** 1970. The isolation of mutants not accumulating poly-β-hydroxybutyric acid. Arch. Mikrobiol. **71:**283–294.
31. **Schubert, P., A. Steinbüchel, and H. G. Schlegel.** 1988. Cloning of the *Alcaligenes eutrophus* poly-β-hydroxybutyrate synthetic pathway and synthesis of PHB in *Escherichia coli*. J. Bacteriol. **170:**5837–5847.
32. **Shimamura, E., K. Kasuya, G. Kobayashi, T. Shiotani, Y. Shima, and Y. Doi.** 1994. Physical properties and biodegradability of microbial poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). Macromolecules **27:**878–880.
33. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering. Transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
34. **Slater, S. C., W. H. Voige, and D. E. Dennis.** 1988. Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. J. Bacteriol. **170:**4431–4436.
35. **Steinbüchel, A.** 1996. PHB and other polyhydroxyalkanoic acids. *In* H. J. Rehm and G. Reed (ed.), Biotechnology. VCH Publishers, Weinheim, Germany.
36. **Steinbüchel, A., E. Hustede, M. Liebergesell, U. Pieper, A. Timm, and H. Valentin.** 1992. Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. FEMS Microbiol. Rev. **103:**217–230.
37. **Steinbüchel, A., and H. E. Valentin.** 1995. Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol. Lett. **128:**219–228.
38. **Steinbüchel, A., and S. Wiese.** 1992. A *Pseudomonas* strain accumulating polyesters of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids. Appl. Microbiol. Biotechnol. **37:**691–697.
39. **Timm, A., and A. Steinbüchel.** 1992. Cloning and molecular analysis of the poly(3-hydroxyalkanoic acid) gene locus of *Pseudomonas aeruginosa* PAO1. Eur. J. Biochem. **209:**15–30.
40. **Valentin, H. E., E. Y. Lee, C. Y. Choi, and A. Steinbüchel.** 1994. Identification of 4-hydroxyhexanoic acid as a new constituent of biosynthetic polyhydroxyalkanoic acids from bacteria. Appl. Microbiol. Biotechnol. **40:**710–716.
41. **Valentin, H. E., A. Schönebaum, and A. Steinbüchel.** 1996. Identification of 5-hydroxyhexanoic acid, 4-hydroxyheptanoic acid and 4-hydroxyoctanoic acid as new constituents of bacterial polyhydroxyalkanoic acids. Appl. Microbiol. Biotechnol. **46:**261–267.
42. **Valentin, H. E., and A. Steinbüchel.** 1993. Cloning and characterization of the *Methylobacterium extorquens* polyhydroxyalkanoic-acid-synthase structural gene. Appl. Microbiol. Biotechnol. **39:**309–317.
43. **Valentin, H. E., and A. Steinbüchel.** 1994. Application of enzymatically synthesized short-chain-length hydroxy fatty acid coenzyme A thioesters for assay of polyhydroxyalkanoic acid synthases. Appl. Microbiol. Biotechnol. **40:**699–709.

Downloaded from http://jb.asm.org/ on September 20, 2020 by guest